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(54) Title: HEPATITIS B VIRUS (HBV) ANTIGENIC POLYPEPTIDE-HEAT SHOCK PROTEIN COMPLEX AND USE THEREOF

(54) 发明名称: 乙肝病毒抗原多肽与热休克蛋白的复合物及其应用

(57) **Abstract:** The present invention provides a kind of HBV-antigen bound to heat shock protein which comprises core antigen, surface antigen and polymerase antigen. The present invention also provides a complex of HBV antigen bound to heat shock protein gp96 and hsp78, as well as a method for preparing the complex. The complex includes a complex of gp96 and hsp78 non-covalently bound to antigenic polypeptide, as well as a fusion protein of the both which results from covalent binding. Such complex can be used to prepare therapeutic vaccine for treating hepatitis B and secondary hepatoma.

(57) 摘要

本发明提供了一类与热休克蛋白结合的乙肝病毒抗原，包括核心抗原、表面抗原和聚合酶抗原。本发明还提供了乙肝病毒抗原与热休克蛋白 gp96 和 hsp78 的复合物及其制备方法，复合物包括 gp96 和 hsp78 与抗原多肽以非共价键结合的复合体，和二者以共价键连接形成的融合蛋白。这种复合物可用于制备治疗乙肝及乙肝继发性肝癌的治疗性疫苗。

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乙肝病毒抗原多肽与热休克蛋白的复合物及其应用

技术领域

本发明涉及乙肝病毒抗原多肽与热休克蛋白gp96和hsp78的复合物及其应用。

技术背景

热休克蛋白(hsp) gp96 (glycoprotein 96)又称作grp94 (glucose-regulated protein 94)，是位于细胞内质网膜上hsp90家族中的成员，分子量约96 KDa，在细胞蛋白折叠和运输过程中发挥重要作用。近几年研究发现它还存在于某些癌细胞表面。gp96分子含有2个保守区，C端区为多肽结合区，能结合5-25个氨基酸的多肽序列，N端区能结合ATP，具有ATP酶活性。热休克蛋白hsp78是细胞质中hsp70家族中的成员，分子量约78kDa，在细胞蛋白折叠和运输过程中发挥重要作用。hsp78分子作为分子伴侣能与细胞中各种短肽结合，它有二个功能区：N端区有ATP酶活性，C端区结合多肽底物。

近年来研究发现从肿瘤组织或病毒感染的细胞中分离纯化的gp96和hsp78分子免疫动物后能引起机体产生针对该肿瘤或病毒的特异性免疫排斥反应，进一步研究表明gp96和hsp78能结合细胞内产生的全部肽库，其中包括抗原多肽，这种特异性免疫反应取决于gp96分子和hsp78分子结合的多肽而非热休克蛋白本身，来自肿瘤或病毒感染的细胞中的gp96分子和hsp78分子通常结合有肿瘤或病毒特异性多肽，gp96分子和hsp78分子能将其结合的抗原肽呈递给主要组织相容性抗原(MHC)分子，从而激活细胞毒性T细胞(CTL)引发机体产生细胞免疫反应。由于gp96和hsp78在细胞抗原呈递过程中发挥重要作用，因此gp96-多肽复合物和hsp78-多肽复合物可用于防治自体肿瘤和某些传染性疾病。

美国纽约大学的Pramod K. Srivastava在自己的研究基础上分别申请了6项美国专利（专利号为6017544，6017540，6007821，5837251，5830464），这些专利主要内容是利用热休克蛋白（hsp）与非共价结合的抗原分子二者形成的复合物对原发性和已转移的肿瘤以及传染性疾病进行治疗，激发机体产生免疫反应，其中抗原分子既包括来自于体内与hsp结合的多肽，又包括在体外能与hsp形成复合物的体外抗原或免疫原性片段。Hsp主要包含hsp70，hsp90和gp96蛋白。

目前已证实gp96和hsp70分子能结合泡状口炎病毒抗原区肽、鼠$H\text{-}2K^b$限制

的卵清蛋白抗原表位肽和 L^d 限制的白血病 CTL 表位肽等抗原多肽。但目前为止未见从病毒感染的病人组织中分离鉴定与 hsp 结合的抗原多肽。

据估计全世界有 3.5 亿人感染乙型肝炎病毒(HBV)，我国约有 1.2 亿，HBV 是导致慢性肝炎、肝硬化和肝癌的首要原因，因此它是一类严重危害我国人民生命健康的传染病。由乙型肝炎继发肝癌的几率也很高，乙肝病毒核心抗原（HBcAg）在肝癌内和肝癌周围组织中的阳性率分别为 62.5%和 29.2%。CTL 引发的细胞免疫反应是机体清除病毒和治愈乙肝的主要途径，在乙肝感染的病人体内 HBV 抗原多肽在肝细胞中被加工后呈递给 I 类 MHC 分子，激活特异 CTL 引发细胞免疫反应，目前已鉴定出人的一些 HBV 核心蛋白上的 CTL 表位，包括 HLA-A2 限制的 HBcAg18-27，HLA-A11 限制的 HBcAg88-96 等。因此研制预防和治疗乙肝和乙肝继发性肝癌的新型药物，尤其是能主动激发机体产生 CTL 免疫反应的药物具有非常重要的意义。

发明公开

本发明的一个目的是提供一种与 gp96 和 hsp78 结合的乙肝病毒抗原的复合物。所述的乙肝病毒抗原可以分别是乙肝病毒核心蛋白上第 88 至第 94 位的氨基酸序列，其序列可以是 YVNTNMG，或其变异序列；乙肝病毒核心蛋白上第 88 至 96 位的氨基酸序列，其序列可以是 YVNTNMGLK，或其变异序列；乙肝病毒核心蛋白上第 141 至 151 位的氨基酸序列，其序列可以是 STLPETTVVRR，或其变异序列；乙肝病毒核心蛋白上第 18 至 27 位的氨基酸序列，其序列可以是 FLPSDFFPSV，或其变异序列；乙肝病毒表面蛋白上第 313 至 321 位的氨基酸序列，其序列可以是 IPIPSSWAF，或其变异序列；乙肝病毒表面蛋白上第 355 至 363 位的氨基酸序列，其序列可以是 WLSLLVPFV，或其变异序列；乙肝病毒聚合酶蛋白上第 575 至 583 位的氨基酸序列，其序列可以是 FLLSLGIHL，或其变异序列。

所述的变异序列是指所述的乙肝病毒抗原通过一个或多个氨基酸的替换，缺失，增加或者侧链的修饰而得到的，同时具有所述的乙肝病毒抗原性的序列。

本发明的复合物既包括热休克蛋白以非共价键与多肽结合，又包括热休克蛋白以共价键与多肽结合。

本发明的再一个目的是提供一种制备本发明的如上所述乙肝病毒抗原多肽与热休克蛋白 gp96 和 hsp78 的复合物的方法。

本发明的又一个目的是提供本发明的这些复合物在制备治疗乙肝及乙肝继发性肝癌的药物中的应用。

本实验室首次从六例 HBV 感染的人肝癌组织中与热休克蛋白 gp96 结合的多肽中分离出一特异 7 肽，经氨基酸序列分析为“YVNTNMG，或 YVNVNMG”，经查询发现该序列位于 HBV 核心蛋白 88-94 位（HBcAg88-94），人工合成该序列并与体外表达的 gp96 蛋白组装，体外合成 gp96-7 肽复合物，将 7 肽与 gp96-7 肽复合物免疫小鼠，均能刺激小鼠产生特异性细胞毒性 T 细胞（CTL），gp96-7 肽复合物免疫原性比单独使用肽高 200 倍以上。实验结果表明 gp96-7 肽复合物可开发成为一种新型乙型肝炎及其继发性肝癌的治疗性药物。

使用人工合成 7 肽“YVNTNMG，或 YVNVNMG”和 N 端带荧光素标记的 7 肽，在体外建立 gp96 蛋白与荧光素标记的 7 肽二者结合的反应体系，根据反应公式

$$\mathrm{G+P} \underset{k_2}{\overset{k_1}{\text{---------}}} \mathrm{GP},$$

其中 G 代表 gp96 蛋白，P 代表 7 肽， GP 代表 gp96 蛋白-7 肽复合物，k_1 、k_2 为反应正反方向的速度常数。该结合反应的平衡常数 k 可由以下关系式得出：

$$k= k_1/k_2=[\mathrm{GP}]/[\mathrm{G}][\mathrm{P}]$$

[GP]、[G]和[P]分别表示反应产物 GP 和底物 G、P 的浓度。确定结合反应体系最佳的温度、盐浓度、pH 值、添加剂与催化剂、gp96 蛋白与 7 肽最佳反应浓度比等因子，测定反应常数，构建最佳反应体系。

在最佳反应体系条件下，体外合成 gp96 蛋白-7 肽复合物。

本发明还提供了一种制备乙肝病毒抗原 7 肽与热休克蛋白 gp96 的复合物的方法，包括将浓度为 0.1-0.15μmol/L 的 gp96 蛋白和浓度为 2.5-3.5μmol/L 的乙肝病毒抗原在含有 5-10%(v/v)甘油的浓度不高于 100mmol/L 的低盐缓冲液中，在 30-39℃下反应 10-30 分钟。

在本发明的方法中，反应温度最好为 37℃，反应时间最好为 15 分钟。

在本发明的方法中，gp96 蛋白的浓度最好为 0.12μmol/L，乙肝病毒抗原的浓度最好为 3.0μmol/L。

本发明还提供了该乙肝病毒抗原 7 肽与热休克蛋白 gp96 以共价键形成的融合蛋白。人工合成对应于该肽的核酸序列，采用分子生物学常用的方法将该序列与 gp96 基因 5’端连接后在大肠杆菌中融合表达。例如，引入限制性酶切位点 BglII 将该 7 肽的核酸序列与 gp96 基因 5’端用 T4 连接酶连接，连接产物的 5’和 3’端引入 2

个限制性酶切位点 BamHI 和 SacI，连接到表达载体 pET30a 中在大肠杆菌中表达，表达产物是 gp96 与 7 肽的融合蛋白。

本发明的 gp96 蛋白-7 肽复合物，包括上述 gp96 以非共价键与 7 肽体外结合形成的复合物，和上述 gp96 以共价键与 7 肽结合形成的融合蛋白，可以使用任何一种公知的免疫方式进行免疫，例如，通过皮下注射、皮内注射、腹腔注射等。gp96 蛋白-7 肽复合物免疫剂量可以是，例如 0.01 nmol、0.05 nmol、0.10 nmol 和 0.50 nmol。当 7 肽被单独用于免疫时，免疫剂量可以为 0.2 nmol、2 nmol、20 nmol。

gp96 蛋白-7 肽复合物和 7 肽在被用于免疫时可以不使用佐剂，或者使用任何公知的佐剂，例如弗氏佐剂、铬明矾等。

本实验室还人工合成了 9 肽"YVNTNMGLK",按照上述最佳反应体系体外合成 gp96 蛋白-9 肽复合物，使用任何一种公知的免疫方式进行免疫，例如皮下注射、皮内注射、腹腔注射等，gp96 蛋白-9 肽复合物免疫剂量可以是，例如 0.01 nmol, 0.05 nmol, 0.10 nmol 和 0.50 nmol。当 9 肽被单独用于免疫时，免疫剂量可以为 0.2 nmol、2 nmol、20 nmol。免疫时可以不使用佐剂，或者使用任何公知的佐剂，例如弗氏佐剂、铬明矾等。gp96-9 肽复合物和 9 肽用于免疫小鼠后均能刺激小鼠产生特异性细胞毒性 T 细胞（CTL)。gp96-9 肽复合物免疫原性比单独使用肽高 300 倍以上。实验结果表明 gp96-9 肽复合物可开发成为一种新型乙型肝炎和乙肝继发性肝癌的治疗性药物。

本实验室还人工合成了 11 肽"STLPETTVVRR"， 10 肽"FLPSDFFPSV"; 9 肽"IPIPSSWAF"; 9 肽"WLSLLVPFV"; 和 9 肽"FLLSLGIHL"。按照上述最佳反应体系体外合成 gp96 蛋白-多肽复合物，使用任何一种公知的免疫方式分别进行免疫，例如皮下注射、皮内注射、腹腔注射等，gp96 蛋白-多肽肽复合物免疫剂量可以是，例如 0.01 nmol, 0.05 nmol, 0.10 nmol 和 0.50 nmol。当多肽被单独用于免疫时，免疫剂量可以为 0.2 nmol、2 nmol、20 nmol。免疫时可以不使用佐剂，或者使用任何公知的佐剂，例如弗氏佐剂、铬明矾等。gp96-多肽复合物和多肽用于免疫小鼠后均能刺激小鼠产生特异性细胞毒性 T 细胞（CTL)。gp96-多肽肽复合物免疫原性比单独使用肽高均在 150 倍以上。实验结果表明 gp96-多肽肽复合物可开发成为一种新型乙型肝炎和乙肝继发性肝癌的治疗性药物。

本实验室还将上述合成的多种多肽，包括"YVNTNMG"; "YVNTNMGLK"; "STLPETTVVRR"; "FLPSDFFPSV"; "IPIPSSWAF"; "WLSLLVPFV"; "FLLSLGIHL"。按照上述最佳反应体系体外合成 hsp78 蛋白-多肽复合物，使用任何一种公知的免疫方式

分别进行免疫，例如皮下注射、皮内注射、腹腔注射等，hsp78 蛋白-多肽复合物免疫剂量可以是，例如 0.01 nmol, 0.05 nmol, 0.10 nmol 和 0.50 nmol。当 7 肽被单独用于免疫时，免疫剂量可以为 0.2 nmol、2 nmol、20 nmol。免疫时可以不使用佐剂，或者使用任何公知的佐剂，例如弗氏佐剂、铬明矾等。hsp78-多肽复合物和多肽用于免疫小鼠后均能刺激小鼠产生特异性细胞毒性 T 细胞（CTL）。hsp78-多肽复合物免疫原性比单独使用肽高均在 150 倍以上。实验结果表明 hsp78-多肽肽复合物可开发成为一种新型乙型肝炎和乙肝继发性肝癌的治疗性药物。

附图简要说明

图 1. 以 7 肽和 gp96-7 肽复合物免疫小鼠 BALB/cJ(H-2^d) 7 天后引发的特异性 CTL 反应。 效应细胞 CTL 对特异性靶细胞的裂解百分率以 4 小时标准 ^{51}Cr 释放测定，效应细胞与靶细胞之比分别是 4、8、12、25、50 和 100，图中裂解率为 10 只小鼠平均值。

实施例

实施例 1 从肝组织中纯化 gp96 蛋白

将三份 HBV 感染的肿瘤组织和一份未感染的正常组织匀浆后离心，用 50%-70% 的 $(NH_4)_2SO_4$ 沉淀，沉淀溶解后采用 ConA Sepharose（Pharmacia 公司）进行亲和层析，结合的蛋白用 10%的α-甲基葡萄糖苷洗脱，洗脱液上 POROS 20QE（PE 公司 BioCAD 灌注层析系统）进行阴离子层析，经过这三步纯化获得 >95%纯度的 gp96 蛋白。gp96 蛋白用 gp96/grp94 单克隆抗体（NeoMarkers 公司）进行 Western 鉴定。其纯度用 SDS-PAGE、银染和反相 HPLC 鉴定。

实施例 2 从 gp96 蛋白释放非共价结合的多肽

将纯化的 gp96 蛋白加入三氟乙酸（TFA）使其终浓度达到 0.2%(pH 约 2.0)，然后用超滤法（分子截留为 10KDa，Pall Filtron 公司）分离多肽，将多肽混合物进行 MALDI-TOF 质谱（PE 公司 Voyager-DE 系统）分析，多肽分子量大多在 600-1100 Da 之间。

实施例 3 反相 HPLC 分析多肽

多肽混合物冻干后溶于溶液 A（0.065%TFA, 2%乙腈），上样于 C18 反相柱（Sephasil peptide C18； 5 nm 粒度；4.6×250nm，Pharmacia 公司），从 0 至 65%溶液 B（0.05%TFA，100%乙腈）进行梯度洗脱，流速为 1ml/min，用 214nm 波长检测。比较肝癌组织和正常组织 HPLC 图谱，发现一个三份肝癌组织共有而正常组织中没有的肽峰。

实施例 4 多肽微量测序与序列分析

收集该特异肽峰用 MALDI-TOF 质谱（PE 公司 Voyager-DE 系统）鉴定其纯度，只发现分子量为 798 Da 的单一峰。对该肽微量测序（Procise™ 491 蛋白测序仪，PE 公司），其氨基酸序列为“YVNTNMG，或 YVNVNMG”，三份肝癌组织得到的序列一致。将该序列在 PubMed 网上蛋白数据库（http://www.ncbi.nlm.nih.gov）查询，发现它位于 HBV 核心蛋白 88-94 位。

实施例 5 gp96 基因克隆

采用宝生物工程（大连）有限公司的 RNA 提取试剂盒(Catrimox-14™ RNA Isolation Kit Ver.2.11)从 0.5mL 经 42°C 热激 2 小时的人血液中用异硫氰酸胍法提取总 RNA,步骤按试剂盒说明。

采用逆转录-聚合酶链式反应（RT-PCR）克隆人 gp96 基因，使用宝生物工程（大连）有限公司的 RT-PCR 试剂盒(TaKaRa RNA LA PCR™ Kit AMV Ver.1.1)，步骤按试剂盒说明，所用引物为：

引物 1：5’ CCGGATCCGAACTTGATGTGGATGGTACA 3’

引物 2：5’ CCGAGCTCCCAAATGGTGAGAGTATAATTTAC 3’

PCR 反应条件如下：94°C 4 分钟；94°C 50 秒，55°C 50 秒，72°C 3 分钟，共 30 循环；72°C 5 分钟。

PCR 产物为约 2.4 Kd 的片段，片段的 5’端和 3’端人为引入二个酶切位点 BamHI 和 SacI，将扩增片段进行测序，gp96 基因包含 2343 bp，碱基序列与文献报导（Maki, RG, et al. GeneBank, 编号：003299）一致。

实施例 6 gp96 基因及 gp96 基因与乙肝病毒抗原多肽核酸的连接片段在大肠杆菌中表达与蛋白纯化

将扩增片段经 BamHI 与 SacI 酶切后连接到表达载体 pET30a(+)转化大肠杆菌 BL21。同时人工合成下列多肽对应的核酸序列："YVNTNMG"；"YVNTNMGLK"；"STLPETTVVRR"；"FLPSDFFPSV"；"IPIPSSWAF"；"WLSLLVPFV"；"FLLSLGIHL"。并在上述核酸序列两端引入限制性酶切位点 BglII，插入到含有 gp96 基因的 pET30a(+)中。

将构建的载体经 1mM IPTG 诱导 4 小时后产物表达，以 10% SDS-PAGE 电泳和考马斯亮蓝染色检测表达产物分子量约 100 Kda，与理论值基本一致。

将 gp96 蛋白和 gp96 蛋白与乙肝病毒抗原多肽的融合蛋白用 Ni 亲和层析柱（Pharmacia 公司）亲和层析和 POROS 20QE（PE 公司 BioCAD 灌注层析系统）进行阴离子层析纯化，以 10% SDS-PAGE 电泳和银染鉴定纯度，得到大于 95%纯度的 gp96 蛋白及其融合蛋白。表达的 gp96 蛋白产物和 gp96 蛋白与乙肝病毒抗原多肽的融合蛋白用 gp96/grp94 单克隆抗体（NeoMarkers 公司）进行 Western 鉴定。

实施例 7 gp96 蛋白与人工合成的多肽体外组装条件试验

人工合成 N 端带有荧光素标记的 7 肽"YVNTNMG，或 YVNVNMG"（委托赛百盛生物工程公司合成），将 gp96 蛋白与多肽体外组装，进行以下一系列试验以确立最佳反应条件：

反应体系温度试验：55°C、39°C、37°C、30°C、或 28°C

反应体系盐浓度试验：低盐缓冲液（20 mmol/L HEPES, pH7.9, 20、100 或 500 mmol/L NaCl, 2 mmol/L $MgCl_2$），高盐缓冲液（20 mmol/L HEPES, pH7.9, 0.5、1.0、或 2.2 mol/L NaCl, 2 mmol/L $MgCl_2$）

反应体系 pH 值试验：pH6.0、pH7.0、pH7.9 和 pH9.0

反应体系添加剂与催化剂：10 mmol/L ADP, 10 mmol/L ATP, 和 10%(V/V)甘油

gp96 蛋白与 7 肽最佳反应浓度及其浓度比率：反应体系 7 肽浓度分别为 0.5、1.0、1.5、2.0、2.5、3.0、3.5 μmol/L，反应体系 gp96 蛋白浓度分别为 0.06、0.09、0.12、0.15、0.18、0.21 μmol/L。测定反应常数 k。gp96 蛋白含量由以下公式计算，$c=1.45A_{280}-0.74A_{260}$，c 为蛋白浓度，$A_{280}$ 和 A_{260} 分别为 280nm 和 260nm 光吸收。荧光素标记的 7 肽含量以 378nm 处的吸收值计。结合反应后用超滤法（分子截留为 10KDa, Pall Filtron 公司）去除未结合的 7 肽，gp96 蛋白-7 肽复合物的含量以 378nm 处的吸收值计。

实施例 8 gp96 蛋白与人工合成的多肽体外组装最佳反应体系构建

通过多组分方差分析优化反应条件，确定最佳反应条件。

人工合成 7 肽“YVNTNMG，或 YVNVNMG”（委托赛百盛生物工程公司合成），建立最佳体外结合反应体系：20 mmol/L HEPES, pH7.9, 20 mmol/L NaCl, 2 mmol/L $MgCl_2$, 10%(V/V)甘油，3.0 μmol/L 7 肽，0.12 μmol/L gp96 蛋白，37℃反应 15 分钟，超滤法（分子截留 10KDa，Pall Filtron 公司）去除未结合的多肽。通过分析测定 gp96 与 7 肽在体外有较高的亲和力，该结合反应的平衡常数 k 约为 5-10。

实施例 9 gp96 蛋白-7 肽稳定性分析

将体外组装的 gp96 蛋白-7 肽复合物于磷酸缓冲液（PBS）中 4°C、-20°C、-70°C 保存均不稳定，以 10% SDS-PAGE 电泳和银染鉴定，30 天后 gp96 蛋白开始形成二聚体或多聚体。冷冻干燥后于 4°C 保存，复合物稳定性较好，90 天后用 10% SDS-PAGE 电泳和银染以及反相 HPLC 检测，其降解率<5%。

实施例 10 免疫小鼠

选用生长 12-16 周的 BALB/cJ 小鼠（$H-2^d$）用于本实验。

免疫方式采用将样品溶于 100 μl 的 PBS 中颈背皮下注射。皮内注射虽然需要的免疫剂量较低，但不易操作，故没有采用。腹腔注射则要求免疫剂量较大，约需 0.5 nmol 才有效，故也没有采用。

gp96 蛋白-7 肽复合物的最适免疫剂量为 0.1 nmol。

免疫时间为第一次免疫一周后进行第二次免疫效果优于免疫一次的效果。

免疫佐剂使用实验发现 gp96 蛋白-7 肽复合物使用弗氏不完全佐剂（FIA）、弗氏完全佐剂、使用铬明矾不仅没有免疫增效作用，而且使其免疫原性降低，原因可能佐剂对 gp96 蛋白-7 肽复合物结构有破坏作用。对于 7 肽使用弗氏佐剂、使用铬明矾均能显著提高其免疫活性，弗氏佐剂效果优于铬明矾。

因此采用皮下注射免疫，将 7 肽溶于缓冲液(90%PBS,10%DMSO/0.1% TFA),gp96-7 肽复合物溶于 PBS 中，注射前剧烈振荡 1 分钟，每支小鼠免疫剂量 7 肽分别为 0.2 nmol,2 nmol 和 20 nmol，将肽与弗氏佐剂乳化后免疫小鼠。 gp96-7 肽复合物免疫剂量分别是 0.01nmol，0.05nmol，0.10 nmol 和 0.50nmol,采用颈背皮下注射，第一

次免疫一周后进行第二次加强免疫，7 天后进行细胞毒性分析。每一处理使用 10 只小鼠。

实施例 11 细胞毒性（CTL）分析

小鼠免疫 7 天后，从每只小鼠收获得约 3×10^7 脾细胞悬于含有 10mM HEPES 缓冲液、5×10^{-5}M 巯基乙醇，抗生素和 10%（V/V）FCS 培养液中，于培养瓶中与经幅射（4500 Rad）的同源 LPS-刺激的 B 淋巴细胞（3：1）和 1 μg/ml 肽在完全培养基中 37℃培养。6 天后收集脾细胞进行 4 小时标准 ^{51}Cr 释放实验(具体方法见 Kuhrober, A, et al. 1997. International Immunology, 9(8):1203-1212）测定细胞毒性活性。简而言之，靶细胞用 10ug/ml 肽于 37℃致敏 30 分钟后加入不同数量的效应细胞，反应体系为 100 ul 的完全培养基。37℃共培养 4 小时后收集上清测定特异裂解率。

从 ^{51}Cr 释放实验可以看出 7 肽和 gp96-7 肽复合物均可刺激小鼠产生特异性细胞毒性 T 细胞，但 gp96-7 肽复合物免疫原性为 7 肽的 200 倍以上，每只小鼠免疫 0.1 nmol（约 10ug）的 gp96-7 肽复合物即能诱发机体产生强烈的细胞免疫反应,细胞毒性测定靶细胞的裂解率在 60%以上。实验结果表明 gp96-7 肽复合物可开发成为一种新型抗 HBV 感染和 HBV 感染的肝癌的治疗药物。

实施例 12. 其它 gp96- 多肽复合物的免疫活性测定

除上述抗原 7 肽之外，我们又选取其它 6 个乙肝病毒抗原多肽与 gp96 体外组装并测定其免疫活性，这 6 个抗原多肽分别是（1）核心抗原多肽“YVNTNMGLK”（位于核心蛋白氨基酸序列 88-96 位）；（2）核心抗原多肽“STLPETTVVRR”（位于核心蛋白氨基酸序列 141-151 位）；（3）核心抗原多肽“FLPSDFFPSV”（位于核心蛋白氨酸酸序列 18-27 位）；（4）表面抗原多肽“IPIPSSWAF”（位于表面蛋白氨基酸序列 313-321）；（5）表面抗原多肽“WLSLLVPFV”（位于表面蛋白氨基酸序列 355-343）；（6）聚合酶抗原多肽“FLLSLGIHL”（位于聚合酶蛋白氨基酸序列 575-583）。分别人工合成这 6 种多肽，采用实施例 8 中所述的最佳反应体系在体外与 gp96 结合，这 6 种多肽与 gp96 均有较高的亲和力，通过测定结合反应平衡常数 K，6 种肽与 gp96 结合反应中 K 值均在 5 以上。

采用实施例 10 中所述的方法用上述 6 种乙肝病毒抗原多肽与 gp96 形成的复合物，和上述 gp96 与 7 种乙肝病毒抗原多肽的融合蛋白分别免疫小鼠，并采用实施例 11

中所述的方法分别对这 6 种复合物进行 CTL 分析，从 51Cr 释放实验可以看出这 6 种乙肝病毒抗原多肽与 gp96 形成的复合物均可刺激小鼠产生特异性细胞毒性 T 细胞，6 种多肽与 gp96 形成的复合物的免疫活性比单独多肽高 150-300 倍。每只小鼠免疫剂量为 0.1nmol(约 10μg)时即能诱发机体产生强烈的细胞免疫反应，通过对这 6 种多肽与 gp96 形成的复合物的细胞毒性测定发现靶细胞的裂解率在 60%-85%之间。

以上实验结果表明 gp96 与乙肝病毒抗原多肽体外组装合成的复合物可开发成为一种新型抗 HBV 感染和 HBV 感染的肝癌的治疗药物。由于实验条件所限本发明专利不可能对每一种乙肝病毒抗原多肽进行体外组装及免疫活性测定，但我们通过选取 7 种有代表性的乙肝病毒核心抗原、表面抗原及聚合酶抗原作研究对象，在体外与 gp96 结合并进行免疫活性测定，大量实验表明 gp96 与这些乙肝病毒抗原多肽形成的复合物均能刺激小鼠产生强烈的免疫反应，gp96 可作为乙肝病毒抗原多肽的新型良好佐剂，可开发成为一种新型治疗疫苗。因此，除上述 7 种乙肝病毒抗原多肽之外，其它任何乙肝病毒抗原多肽与 gp96 结合作为新型疫苗也应当在本发明的保护范围之内。

实施例 13. 热休克蛋白 hsp70 基因克隆与表达

采用宝生物工程（大连）有限公司的 RNA 提取试剂盒（Catlimox-14TM RNA Isolation Kit Ver2.11）从 200mg 的人肝癌组织中用异硫氰酸法提取总 RNA，步骤按试剂盒说明。

采用逆转录一聚合酶链式反应（RT-PCR）克隆人 hsp70 家族中 hsp78，使用宝生物工程（大连）有限公司的 RT-PCR 试剂盒，步骤按试剂盒说明。

所用引物为：

引物 1：GG GGATCC ATG AAG TTC ACT GTG GTG GCG GCG

引物 2：GG GTCGAC CTA CTA CTC ATC TTT TTC TGA TGT

PCR 反应条件如下：94°C 4 分钟；94°C 50 秒，55°C 50 秒，72°C 2 分钟，其 30 循环；72°C 5 分钟。

PCR 产物为约 2.0kb 的片段，片段的 5' 端和 3' 端人为引入二个酶切位点 BamHI 和 SalI，将扩增片段进行测序，hsp78 基因包含 1965bp，碱基序列与文献报导(Mech. Ageing Dev. 1998, 104(2):149-158)一致。

将扩增片段经 BamHI 与 SalI 酶切连接到表达载体 PET30a(+)转化大肠杆菌 BL21，同

时人工合成下列多肽对应的核酸序列：“YVNTNMG”；“YVNTNMGLK”；“STLPETTVVRR”；“FLPSDFFPSV”；“IPIPSSWAF”；“WLSLLVPFV”；“FLLSLGIHL”。并在上述核酸序列两端引入限制性酶切位点 BglII，插入到含有 hsp78 基因的 pET30a(+)中。

将构建的载体经 1mM IPTG 诱导 4 小时后表达，以 10% SDS-PAGE 电泳和考马斯亮蓝染色检测表达产物分子量约 80 kDa，与理论值基本一致。

将表达产物用 ADP-Agarose 亲和层析（Sigma）和 MonoQ(Pharmacia)阴离子层析纯化，以 10% SDS-PAGE 电泳和考马斯亮蓝染色（电泳上样量约 5μg）鉴定纯度，得到大于 95%纯度的蛋白。表达的 HSP78 极其融合蛋白用 hsp70 单克隆抗体(Sigma)进行 Western 鉴定。

实施例 14. hsp 78-多肽免疫原性测定

将 hsp78 与 7 种多肽体外组装形成的复合物，最佳反应体系同 gp96（见实施例 8），将体外合成的复合物和 hsp78 与 7 种多肽的融合蛋白免疫小鼠。小鼠免疫方式、免疫剂量以及免疫程序同 gp96（见实施例 10）。细胞毒性（CTL）分析方法同 gp96(见实施例 11)。从 51Cr 释放实验可以看出 hsp78-7 肽复合物可刺激小鼠产生特异性细胞毒性 T 细胞，hsp78-7 肽复合物的免疫原性为单独 7 肽的 150 倍以上，每只小鼠免疫 0.1nmol(约 10μg)即能诱发机体产生强烈的细胞免疫反应，细胞毒性测定靶细胞的裂解率在 50%以上。实验表明 hsp78-7 肽复合物可开发成为一种新型抗 HBV 感染和 HBV 感染的肝癌的治疗药物。

权 利 要 求

1．一种乙肝病毒抗原与热休克蛋白 gp96 或 hsp78 的复合物。

2．按照权利要求 1 所述的复合物，其中，所述的乙肝病毒抗原的氨基酸序列为 YVNTNMG，YVNVNMG，YVNTNMGLK，STLPETTVVRR，FLPSDFFPSV，IPIPSSWAF，WLSLLVPFV，FLLSLGIHL 和/或他们的变异序列。

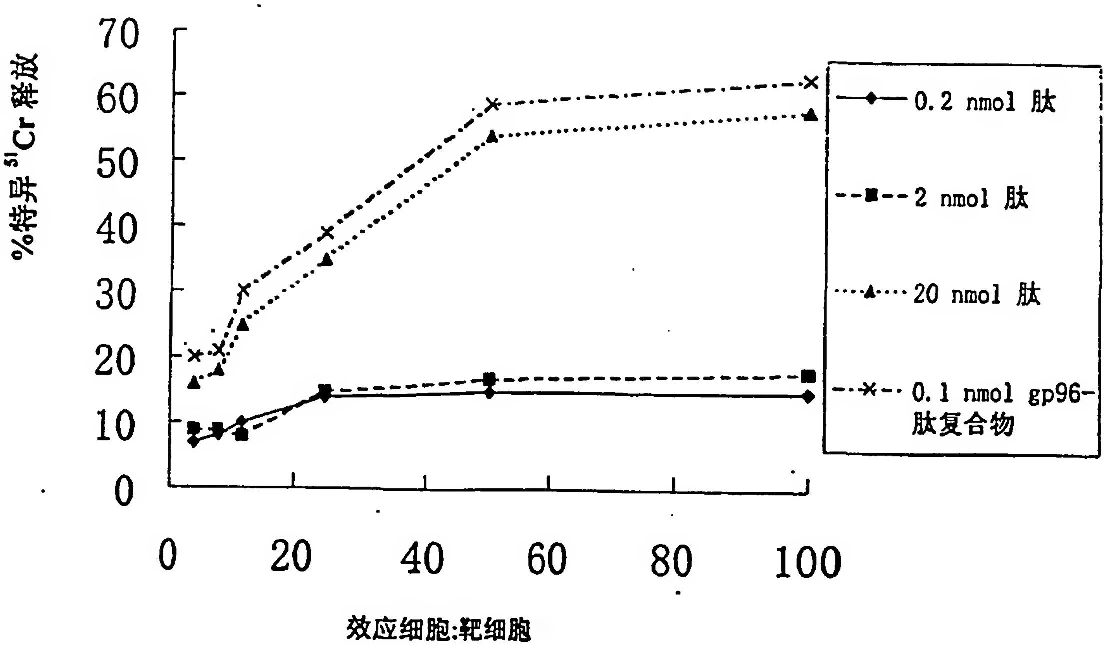
3．按照权利要求 1 所述的复合物，其中，热休克蛋白以非共价键与乙肝病毒抗原结合，或者热休克蛋白以共价键与乙肝病毒抗原结合成融合蛋白。

4．一种制备权利要求 1 所述的乙肝病毒抗原与热休克蛋白 gp96 或 hsp78 的复合物的方法，包括将浓度为 0.1-0.15μmol/L 的 gp96 蛋白和浓度为 2.5-3.5μmol/L 的乙肝病毒抗原在含有 5-10%(v/v)甘油的浓度不高于 100mmol/L 的低盐缓冲液中，在 30-39℃下反应 10-30 分钟。

5．按照权利要求 4 所述的方法，其中，反应温度为 37℃，反应时间为 15 分钟。

6．按照权利要求 4 所述的方法，其中，gp96 蛋白和 hsp78 的浓度均为 0.12μmol/L，乙肝病毒抗原的浓度为 3.0μmol/L。

7．权利要求 1 所述的乙肝病毒抗原与热休克蛋白 gp96 或 hsp78 的复合物在制备治疗乙肝及乙肝继发性肝癌的治疗性疫苗中的应用。



%特异 51Cr 释放
70
60
50
40
30
20
10
0
0
20
40
60
80
100
效应细胞:靶细胞
0.2 nmol 肽
2 nmol 肽
20 nmol 肽
0.1 nmol gp96-肽复合物

图 1

INTERNATIONAL SEARCH REPORT	International application No. PCT/CN01/00295

A. CLASSIFICATION OF SUBJECT MATTER

IPC[7] C07K19/00, C07K14/02, C07K14/47, C07K1/00, A61P1/16

According to International Patent Classification(IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched(classification system followed by classification symbols)

IPC[7] C07K19/00, C07K14/02, C07K14/47, C07K1/00, A61P1/16

Documentation searched other than minimum documentation to the extent that such documents are included in the field searched

Chinese Patnets, Chinese Scientific and Technical Journals

Electronic data base consulted during the international search(name of data base and, where practicable, search terms used)

GenBank, EPOQUE, BA, MEDLINE

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant claim No.
A	CURRENT OPINION IN IMMUNOLOGY, 6(5):728-732, 1994 see the abstract	1－7
A	WO9610411(MOUNT SINAI SCHOOL MEDCINE), 1996 see the abstract	1－7
A	WO9834641(UNIV FORDHAM), 1998 see the abstract	1－7
A	WO9918801(UNIV FORDHAM), 1999 see the abstract	1－7

☐ Further documents are listed in the continuation of Box C. ☒ See patent family annex.

* Special categories of cited documents:

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason(as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
14 June 2001 (14. 06. 01)	28 JUN 2001 (28. 06. 01)

Name and mailing address of the ISA/	Authorized officer
The Chinese Patent Office 6, Xitucheng Road, Haidian District, Beijing, 100088, China	SUN, guangxiu 秀孙印广
Facsimile No. 86-010-62019451	Telephone No. 86-010-62093884

INTERNATIONAL SEARCH REPORT
Information on patent family members

International application No.
PCT/CN01/00295

Patent document cited in search report	Publication date	Patent family members	Publication date
WO9610411	1996-4-14	US6017544	2000-01-25
		AU2281995	1996-04-11
		EP0784477	1997-07-23
		US5750119	1998-05-12
		US5997873	1999-12-07
WO9834641	1998-08-13	AU6145598	1998-08-26
		EP0973548	2000-01-26
		US6017540	2000-01-25
		ZA9800978	1998-10-28
WO9918801	1999-04-22	ZA9809461	1999-06-30
		AU1086299	1999-05-03
		US6007821	1999-12-28

国际检索报告

国际申请号 PCT/CN01/00295

A. 主题的分类

IPC[7] C07K19/00, C07K14/02, C07K14/47, C07K1/00, A61P1/16

按照国际专利分类表(IPC)或者同时按照国家分类和 IPC 两种分类

B. 检索领域

检索的最低限度文献(标明分类体系和分类号)

IPC[7] C07K19/00, C07K14/02, C07K14/47, C07K1/00, A61P1/16

包含在检索领域中的除最低限度文献以外的检索文献

中国专利文献数据库，中文科技期刊数据库

在国际检索时查阅的电子数据库(数据库的名称和，如果实际可行的，使用的检索词)

GenBank, EPOQUE, BA, MEDLINE

C. 相关文件

类 型*	引用文件，必要时，包括相关段落的说明	相关的权利要求编号
A	CURRENT OPINION IN IMMUNOLOGY, 6(5):728-732, 1994 见摘要	1－7
A	WO9610411(MOUNT SINAI SCHOOL MEDCINE), 1996 见摘要	1－7
A	WO9834641(UNIV FORDHAM), 1998 见摘要	1－7
A	WO9918801(UNIV FORDHAM), 1999 见摘要	1－7

☐ 其余文件在 C 栏的续页中列出。 ☒ 见同族专利附件。

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		AU2281995	1996-04-11
		EP0784477	1997-07-23
		US5750119	1998-05-12
		US5997873	1999-12-07
WO9834641	1998-08-13	AU6145598	1998-08-26
		EP0973548	2000-01-26
		US6017540	2000-01-25
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WO9918801	1999-04-22	ZA9809461	1999-06-30
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Dated: July 16, 2002

Kara Dempsey

Kara Dempsey
Vice President
Merrill Corporation

Sworn to and signed before
Me this 16th day of
July, 2002

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Please refer to "BRIEF EXPLANATION OF CODES AND ABBREVIATIONS" in the beginning pages of each issue of PCT Gazette for meanings of two-letter codes and other abbreviations.

(54) Title: HEPATITIS B VIRUS (HBV) ANTIGENIC POLYPEPTIDE-HEAT SHOCK COMPLEX AND USE THEREOF

(57) ABSTRACT: The present invention provides a kind of HBV-antigen bound to heat shock protein, which comprises core antigen, surface antigen and polymerase antigen. The present invention also provides a complex of HBV antigen bound to heat shock protein gp96 and hsp78, as well as a method for preparing the complex. The complex includes a complex of gp96 and hsp78 non-covalently bound to antigenic polypeptide, as well as a fusion protein of the both which results from covalent binding. Such complex can be used to prepare therapeutic vaccine for treating hepatitis B and secondary hepatoma.

HEPATITIS B VIRUS (HBV) ANTIGENIC POLYPEPTIDE - HEAT SHOCK COMPLEX AND USE THEREOF

Area of Technology

This invention relates to complexes of Hepatitis B Virus and heat shock proteins gp86 and hsp78 and their application.

Background of Technology

The gp96 (glycoprotein 96), also known as grp94 (glucose-regulated protein 94), of which the molecular weight is approximately 96 kDa, is a heat shock protein (hsp), and as a member of the hsp90 family found on endoplasmic reticulum membrane, plays a major role in the folding and translocation of cell proteins. In recent years, studies show that it is also found on the surface of certain cancer cells. A gp96 molecule has two conserved areas, namely a C-terminal end area for binding a polypeptide sequence containing 5 to 25 amino acids, and an N-terminal end area for binding ATP, which is an area of ATP activity. The hsp78, of which the molecular weight is approximately 78kDaa, is a heat shock protein, and as a member of the hsp70 family found in cytoplasm, plays a critical role in the folding and translocation of cell proteins. As a molecular chaperone, hsp78 is able to combine with various short peptides in cells, and has two functional areas: an N-terminal end area of ATP enzyme activity and a C-terminal end area for binding polypeptide substrates.

In recent years, studies indicate that, when used to immunize animals, gp96 and hsp78 molecules separated and purified from tumor tissues or cells infected with a virus will cause organismic immunological rejection that is distinctive to such tumor or virus. Further studies reveal that gp96 and hsp78 are able to combine with all peptide libraries generated in cells, including antigenic polypeptide, and such distinctive immune response depends on the polypeptide generated by binding gp96 molecules with hsp78 molecules, not the heat shock protein itself, where gp96 molecules and hsp78 molecules from tumor or cells infected with a virus are usually bound with polypeptide distinctive to such tumor or virus, and the gp96 molecules and hsp78 molecules may present the antigenic peptide that they carry to majorhistocompatibility complex (MHC) antigen molecules, thus triggering cellular immune response from organism by activating cytotoxic T lymphocyte (CTL). Since gp96 polypeptide complexes and hsp78 polypeptide complexes play a critical role in presenting cell-antigens, gp96 polypeptide complexes and hsp78 polypeptide complexes may be used to prevent and treat autologous tumors and some infectious diseases.

Based on his own work, Pramod K. Srivastava of New York University applied for and obtained six US Patents (Patent No. 6017544, 6017540, 6007821, 5837251, 5830464), which mainly relate to the application of complexes formed by heat shock proteins (hsp)

and non-covalently bound antigen molecules to treat primary tumors and tumors with metastasis by stimulating immune response from the organism, where antigen molecules include both in vivo peptides bound with hsp and in vitro antigens or immunogenic segments that can combine with hsp to form complexes on an in vitro basis. Here, hsp mainly includes hsp70, hsp90 and gp96 proteins.

It has been proven that gp96 molecules and hsp80 molecules can combine with antigenic peptides such as peptides in the antigenic sites of vesicular stomatitis virus, epitope peptides of mice H-2K^b-restricted ovalbumin and Leukemia CTL epitope peptide. However, there has been no reported case of hsp-carrying antigenic polypeptides separated and recognized from the tissue of a patient infected with the virus.

It is estimated that three hundred and fifty million people in the world are infected with the hepatitis B virus (HBV), and one hundred and twenty million of them are in China. As it is the primary cause for chronic hepatitis, cirrhosis and liver cancer, HBV poses a serious threat to the health of the Chinese people. The probability of secondary liver cancer associated with Hepatitis B remains high, and there is a 62.5% chance of having a positive Hepatitis B Core Antigen (HBcAg) test result in the liver cancer zone and a 29.2% chance of having such a result in the peripheral tissues. The cellular immune response triggered by CTL is the primary way for the organism to clear the virus and cure hepatitis B. In the body of a hepatitis B patient, the HBV antigen polypeptide, after being processed in liver cells, is transmitted to I-type MHC molecules to activate distinctive CTL, thus triggering a cellular immune response. Currently, some epitopes have been identified in the HBV core protein, including HLA-A2 restricted HBcAg18-27, and HLA-restricted HBcAg88-96. Therefore, it is of critical importance to develop novel drugs for preventing and treating hepatitis B and secondary liver cancer associated with hepatitis B, especially drugs that can actively stimulate the CTL immune response from the organism.

Invention Publication

One objective of the present invention is to provide a kind of complex formed by combining hepatitis B virus antigen with gp96 and hsp78. Here, the hepatitis B virus antigen may be one of the following: the 88-94 amino acid sequence of the HBV core protein, whose sequence can be YVNTNMG or one of its variants; the 88-96 amino acid sequence of the HBV core protein, whose sequence can be YVNTNMGLK or one of its variants; the 141-151 acid amino sequence of the HBV core protein, whose sequence can be STLPETTVVRR or one of its variants; the 18-27 amino acid sequence of the HBV core protein, whose sequence can be FLPSDFFPSV or one of its variants; the 313-321 amino acid sequence of the HBV surface protein, which can be IPIPSSWAF or one of its variants; the 355-363 amino acid sequence, which can be WLSLLVPFV or one of its variants; the 575-583 amino acid sequence of the HBV polymerase protein, whose sequence can be FLLSLGIHL or one of its variants.

A variant sequence shall mean a sequence obtained by substitution, removal, addition or side chain modification of one or more amino acids of the HBV antigen, while a sequence with the said HBV antigenicity is preserved.

The complexes involved in the present invention include those formed by combining heat shock proteins with polypeptide through non-covalent bonds, and by combining heat shock proteins with polypeptide through covalent bonds.

Another objective of the present invention is to provide a method for preparing such complexes of HBV antigenic polypeptides and heat shock proteins gp96 and hsp78 as those specified in the invention.

One more objective of the present invention is to enable the application of these complexes to the preparation of drugs for treating hepatitis B and secondary liver cancer associated with hepatitis B.

From six samples of human liver cancer tissues infected with HBV, this laboratory, for the first time ever, separated a distinctive 7 peptide out of the polypeptide bound with heat shock protein gp96. The amino acid sequence was analyzed and determined to be YVNTNMG or YVNVNMG, which was found in the study to fit the 88-94 sequence of HBV core protein (HBcAg88-94). Such a sequence has been artificially synthesized and assembled with the in vitro expressed gp96 protein to form the gp96-7 peptide complex. When the 7 peptide and gp96-7 complex were applied to immunize the mice, distinctive cytotoxic T lymphocytes (CTL) were stimulated in the body of the mice, and the immunogenicity of gp96-7 peptide is over 200 times more than the case when only peptide is applied. Experimental results show that gp96-7 peptide complex has the potential to be developed into a new type of therapeutic drug for treating hepatitis B and the associated secondary liver cancer.

The artificially synthesized 7 peptide YVNTNMG or YVNVNMG and the 7 peptide with flourescein marker at the N-terminus end have been used to establish an in vitro reaction system combining gp96 proteins and flourescein-marked 7 peptide. Below is the reaction equation:

$$G + P \underset{K_2}{\overset{K_1}{\rightleftharpoons}} GP$$

where, G is for gp96 protein; P is for 7 peptide; GP is for the gp96 protein-7 peptide complex; and K_1 and K_2 are velocity constants for positive and reverse reactions. The equilibrium constant K for this combination reaction can be obtained from the following relationship:

$$K=K1/K2=[GP]/[G][P]$$

where [GP], [G] and [P] are concentrations of the resultant GP, and substrates G and P. For this combination reaction, an optimal reaction system is constructed by determining factors such as the optimal temperature, salt concentration, pH, additives and catalysts, and the optimal reaction concentration ratio of gp96 protein and 7 peptide, and by measuring the reaction constants.

The gp96-7 peptide complex is then synthesized in vitro under optimal reaction system conditions.

In addition, the present invention provides a method for preparing the complex of HBV antigenic 7 peptide and heat shock protein gp96, including the reaction of 0.1-0.15 μmol /L of gp96 protein with 2.5-3.5 μmol /L of HBV antigen at 30-39℃ for 30-39 minutes in a low salt buffer solution which contains 5-10% glycerin and whose concentration is no more than 100 mmol/L.

According to the present method, the suggested reaction temperature is 37℃ and the suggested reaction time is 15 minutes.

According to the present method, the suggested concentration of the gp96 protein is 0.12 μmol/L, and the suggested concentration of the HVB antigen is 3.0 μmol/L.

Additionally, the present invention provides fusion protein formed by covalently binding HBV antigenic 7 peptide and heat shock protein gp96. The nucleic acid sequence corresponding to such peptide is artificially synthesized and then linked to the 5' end of the DNA encoding gp96 using common methods in molecular biology for fusion expression in colibacillus. For example, -generating a BglII end to link the nucleic acid sequence of such 7 peptide to the 5' end of gp96 with T4 ligase, and introducing BamHI and SacI to the 5' end and 3' end of the resulting construct, which is then cloned into the expression vector pET30a for expression in colibacillus, results in expression of a fusion protein of gp69 and 7 peptide.

The gp96-7 peptide complex in the present invention, including said complex formed by non-covalently binding gp96 with 7 peptide, and said fusion protein formed by covalently binding gp69 with 7 peptide, may be used in immunization through any publicly known immunization methods, such as subcutaneous injection, intradermal injection or abdominal cavity injection. The immunizing dose of gp96-7 peptide complex may be 0.01 nmol, 0.05 nmol, 0.10 nmol or 0.50 nmol. When 7 peptide is used independently in immunization, the immunizing dose may be 0.2 nmol, 2 nmol or 20 nmol.

When gp96-7 peptide complex and 7 peptide are used in immunization, adjuvant is not necessary, or any publicly known adjuvant can be used, such as Freund's adjuvant or chromalum.

This laboratory artificially synthesized the 9 peptide "YVNTNMGLK", and further synthesized in vitro gp96-9 peptide complex under said optimal reaction system conditions, which can be used in immunization through any publicly known immunization methods, such as subcutaneous injection, intradermal injection and abdominal cavity injection. The immunizing dose of gp96-9 peptide complex may be 0.01 nmol, 0.05 nmol, 0.10 nmol or 0.50 nmol. When 7 peptide is used independently in immunization, the immunizing dose may be 0.2 nmol, 2 nmol or 20 nmol. An adjuvant is not necessary in immunization, or any publicly known adjuvant can be used, such as Freund's adjuvant or chromalum. Both gp96-9 peptide complex and 9 peptide stimulate distinctive cytotoxic T lymphocytes (CTL) in mice. The immunogenicity of gp96-9 peptide is over 300 times more than when peptide is applied independently. Experimental results show that the gp96-9 peptide complex has the potential to be developed into a new type of therapeutic drug for treating hepatitis B and the associated secondary liver cancer.

This laboratory artificially synthesized the 11 peptide "STLPETTVVRR"; 10 peptide "FLPSDFFPSV"; 9 peptide "IPIPSSWAF"; 9 peptide "WLSLLVPFV"; and 9 peptide "FLLSLGIHL". The gp96-polypeptide complex was further synthesized in vitro under said optimal reaction system conditions, which can then be used in immunization through any publicly known immunization methods, such as subcutaneous injection, intradermal injection and abdominal cavity injection. The immunizing dose of gp96-polypeptide complex may be 0.01 nmol, 0.05 nmol, 0.10 nmol or 0.50 nmol. When polypeptide is used independently in immunization, the immunizing dose may be 0.2 nmol, 2 nmol or 20 nmol. An adjuvant is not necessary in immunization, or any publicly known adjuvant can be used, such as Freund's adjuvant or chromalum. Both the gp96-polypeptide complex and polypeptide stimulate distinctive cytotoxic T lymphocytes (CTL) in mice. The immunogenicity of gp96-polypeptide is over 150 times more than when peptide is applied independently. Experimental results show that gp96-polypeptide complex has the potential to be developed into a new type of therapeutic drug for treating hepatitis B and the associated secondary liver cancer.

This laboratory also used the said polypeptides, such as "YVNTNMG", "YVNTMGLK", "STLPETTVVRR", "FLPSDFFPSV", "IPIPSSWAF", "WLSLLVPFV" and "FLLSLGIHL" to synthesize hsp78-polypeptide complexes in vitro under the said optimal reaction system conditions, which can then be used in immunization through any publicly known immunization methods, such as subcutaneous injection, intradermal injection and abdominal cavity injection. The immunizing dose of hsp78-polypeptide complex may be 0.01 nmol, 0.05 nmol, 0.10 nmol or 0.50 nmol. When polypeptide is used independently in immunization, the immunizing dose may be 0.2 nmol, 2 nmol or 20 nmol. An adjuvant is not necessary in immunization, or any publicly known adjuvant can be used, such as Freund's adjuvant and chromalum. Both the hsp78 -polypeptide complex and polypeptide stimulate distinctive cytotoxic T lymphocyte (CTL) in mice. The immunogenicity of hsp78-polypeptide is over 150 times more than when peptide is applied independently. Experimental results show that hsp78-polypeptide complex has the potential to be developed into a new type of therapeutic drug for treating hepatitis B and the associated secondary liver cancer.

Brief Description of Illustrations

Figure 1 shows the distinctive CTL response triggered 7 days after immunizing mice BALB/Cj (H-2^d) with 7 peptide and gp96-7 peptide complex. The cleavage percentage of effector cells relative to target cells is measured for 4 hours using standard ^{51}Cr. The ratio of effector cells to target cells is 4, 8, 12, 25, 50 and 100, respectively, and the cleavage percentage indicated is the average for 10 mice.

Examples:

Example 1 Purification of gp96 protein from liver tissue

Three pieces of tumor tissue infected with HBV and one piece of non-infected normal tissue were made into a homogenate. The homogenate was centrifuged and the sediment was dissolved with 50%-80% $(NH_4)_2SO_4$. Then an affinity chromatography was performed on the dissolved sediment with ConA Sepharose (made by Pharmacia). A 10% α-methyl glucoside was used to elute the combined protein, and then an anionic chromatography was performed on the eluate with POROS 20QE (PE's BioCAD perfusion chromatography system). This three-step process resulted in gp96 protein that was more than 95% pure. The gp96/grp94 monoclonal antibody (NeoMarkers) was used to perform a Western test to validate the identity of gp96 protein. The purity was measured using SDS-PAGE, silver staining and reversed phase HPLC.

Example 2 Release of non-covalently bound polypeptide from gp96 protein

The purified gp96 protein was added into trifuoroacetic acid (TFA) to prepare a 0.2% solution (with a pH of about 2.0), and then an ultra filtration was performed to separate polypeptides (Molecular Weight Cut Off: 10kDa, by Pall Filtron). MALDI-TOF mass spectrometry was then applied (using PE's Voyager-DE system) to separate the mixed peptides. It was found that the molecular weight of most of such polypeptides fell between 600-1100 kDa.

Example 3 Analysis of polypeptides using reversed phase HPLC

A lyophilized polypeptide mixture was dissolved in solution A (0.065% TFA, 2% ethanol), and then this sample was loaded on a C18 reversed-phase column (Sephasil peptide C18; 5 nm Particle Size; 4.6×250nm, by Pharmacia) for 0% to 65% gradient elution with solution B (0.05%TFA, 100% acetonitrile), at the flow rate of 1ml/min. Recorded at wavelength 214nm. By comparing the HPLC chromatogram of liver cancer tissue and normal tissue, a peptide peak was detected for all three pieces of liver cancer tissue, while it was not detected for the normal tissue.

Example 4 Micro-sequencing and sequence analysis of polypeptides

The specific peptide peak was collected and MALDI-TOF mass spectrometry (PE's Voyager-DE system) was used to determine its purity, where only one single peak associated with molecular weight of 798 Da was detected. Micro sequencing was performed on the peptide (using Procise™ 491 Protein Sequencer made by PE) and the amino acid sequence was found to be "YVNTNMG" or "YVNVNMG". The same sequence was obtained for all three pieces of liver cancer tissue. A query performed in the PubMed online protein database (http://www.ncbi.nlm.nih.gov) found that the sequence was located at 88-94 segment of HBV core protein.

Example 5 Gene cloning of gp96

The RNA isolation kit (Catrimox-14™ RNA Isolation Kit Ver. 2.11) manufactured by TaKaRa Biotechnology (Dalian) Co., Ltd. was used to isolate the total RNA through the guanidinium isothiocyanate method in 0.5 ml of human blood that was heat-activated at 42 ℃ for 2 hours. Instructions provided with the isolation kit were followed in the process.

Reverse transcriptase-polymerase chain reaction (RT-PCR) was then employed to clone human gp96 genes. The RT-PCR Kit (TaKaRa RNA LA PCR™ Kit AMV Ver. 1.1) manufactured by TaKaRa Biotechnology (Dalian) Co., Ltd. was used and instructions provided with the isolation kit were followed in the process. The following primers were used:
Primer 1: 5' CCGGATCCGAACTTGATGTGGATGGTACA 3'
Primer 2: 5' CCGAGCTCCCAAATGGTGAGAGTATAATTTAC 3"

PCR reaction conditions: 1 minutes at 94℃; 50 seconds at 94℃, 50 seconds at 55℃, 3 minutes at 72℃, for 30 cycles; 5 minutes at 72℃.

The PCR product was a fragment that has about 2.4 Kd. Sequencing was performed on the fragment, which was amplified by artificially introducing two enzyme cutting site BamHI and SacI to the 5' end and the 3' end. It was found that gp96 gene contains 2343 bp, and the base sequencing was consistent with reported results (Maki, RG, et al. GeneBank, No:

003299).

Example 6 Expression of gp96 gene and the fragment formed by linking gp96 gene to the HBV antigen polypeptide nucleic acid in colibacillus and the purification of proteins

The amplified fragment, after restriction using BamHl and Sacl enzymes, was linked to the expression vector pRT30a(+) and transformed into colibacillus BL21. At the same time, nucleic acid sequences associated with the following peptides were artificially synthesized: "YVNTNMG"; "YVNTNMGLK"; "STLPETTVVRR"; "FLPSDFFPSV"; "IPIPSSWAF"; "WLSLLVPFV"; "FLLSLGIHL". In addition, BglII restriction-generating sites were introduced to the two ends of each of the said nucleic acid sequence , and the sequence was then inserted into pRT30a (+) containing the gp96 gene.

Product was expressed after inducing the vector thus constructed with 1mM IPTG for 4 hours. The 10% SDS-PAGE electrophoresis method and eCoomassie brilliant blue staining were used to measure the molecular weight of the expression product, and the result was about 100 kDa, which was basically consistent with theoretical value.

Affinity chromatography with Ni affinity chromatography column (by Pharmacia) was performed on the gp96 protein, the fusion protein formed by the gp96 protein, and the HBV antigen polypeptide. These were purified by anionic chromatography using POROS 20QE (PE's BioCAD perfusion chromatography system). The purity was measured using 10% SDS-PAGE electrophoresis and silver staining. The purity of the gp96 protein and its fusion protein was more than 95%. Western analysis was then performed on the expressed gp96 protein, the fusion protein of gp96 protein, and the HBV antigen polypeptide with gp96/grp94 monoclonal antibody (by NecMarkers). Example 7 Experiment for determining the optimal conditions for the in vitro assembly of gp96 and artificially synthesized polypeptide

The 7 peptide "YVNTNMG" or "YVNVNMG" with a fluorescein-marked N-terminus was artificially synthesized (by Sai Bai Sheng Biotechnology Co., Ltd. on a subcontracting basis). The gp96 protein and polypeptide were then assembled in vitro, and a series of experiments were carried out on them to determine the optimal reaction conditions:

Reaction system temperatures tested: 55°C, 39°C, 37°C, 30°C or 28°C

Reaction system salt concentrations tested: low salt buffer solution (20 mmol/L HEPES, pH 7.9, 20, 100 or 500 mmol/L NaCl, 2mmol/L $MgCl_2$), high salt buffer solution (20 mmol/L HEPES, pH 7.9, 0.5, 1.0 or 2.2 mmol/L NaCl, 2 mmol/L $MgCl_2$)

Reaction system pH values tested: pH6.0, pH7.0, pH7.9 and pH9.0

Additives and catalysts for the reaction system tested: 10 mmol/L ADP, 10 mmol/L ATP and 10% (V/V) glycerine

The optimal reaction concentrations and the concentration ratio for the gp96 protein and

the 7 peptide: for the 7 peptide in the reaction system, the concentrations tested were 0.5, 1.0, 1.5, 2.0, 2.5, 3.0, and 3.5 μ mol/L; for the gp96 protein in the reaction system, the concentrations tested were 0.06, 0.09, 0.12, 0.15, 0.18, and 0.21 μ mol/L. The reaction constant K was then determined. The volume of the gp96 protein contained was calculated using the following formula: $c=1.45A_{280}-0.74A_{260}$, where c was the protein concentration, A_{280} and A_{260} were light absorptions at wavelengths 280nm and 260nm. The volume of the fluorescein-marked 7 peptide contained was measured using the absorption at 378nm. After the reaction, ultra filtration (Molecular Weight Cut Off: 10kDa, by Pall Filtron) was carried out to remove unconjugated 7 peptide. The volume of gp96-7 peptide complex contained was measured using absorption at 378 nm.

Example 8 The construction of an optimal reaction system for in vitro assembly of gp96 and artificially synthesized polypeptide

Multi-component variance analysis was employed to optimize the reaction conditions and determine the optimal reaction conditions.

Through artificial synthesis of the 7 peptide "YVNTNMG" or "YVNVNMG" (synthesized by Sai Bai Sheng Biotechnology Co., Ltd. on a subcontracting basis), an optimal reaction system for in vitro synthesis was established: 20 mmol/L HEPES, pH7.9, 20 mmol/L NaCl, 2 mmol/L $MgCl_2$, 10% (V/V) glycerine, 3.0 μ mol/L 7 peptide, 0.12 μ mol/L gp96 protein, 15 minutes at 37°C, with removal of unconjugated polypeptide through ultra filtration (Molecular Weight Cut Off: 10kDa, Pall Filtron). High in vitro affinity between the gp96 and 7 peptide was proved by analysis and measurement. The equilibrium constant K of this reaction was around 5-10.

Example 9 Stability analysis of the gp96 protein-7 peptide complex

The gp96 protein-7 peptide complex was not stable when stored in phosphate buffered saline solution (PBS) at 4°C, -20°C and -70°C. It was found that the gp96 protein began to form dimers or polymers after 30 days of storage, as shown by 10% SDS-PAGE electrophoresis and silver staining tests. The complex was stable when lyophilized and stored at 4°C. After 90 days of storage, the degradation percentage was less than 5%, as shown by 10% SDS-PAGE electrophoresis, silver staining tests and reversed-phase HPLC.

Example 10 Immunization of Mice

An experiment was carried out on BALB/c mice (H-2^d) aged 12-16 weeks.

Immunization was carried out through nape subcutaneous injection of sample dissolved in 100 μl PBS. Intradermal injection was not employed because it was not an easy process, although it would have required a smaller immunizing dose. Abdominal cavity injection was not employed because it would have required a larger immunizing dose of 0.5 nmol to be effective.

The optimal immunizing dose for the gp96 protein-7 peptide complex was 0.1 nmol.

Timing of immunization: when a second immunization was given one week after the first one, the immunization effect was greater than a single immunization.

In immunizing adjuvant tests, it was found that the use of Freund's Incomplete Adjuvant (FIA), Freund's Complete Adjuvant or chromalum with the gp96-7 peptide complex did

not result in a gain in immunization; instead, it resulted in a lower immunogenicity. A possible reason was that adjuvant might be destructive to the gp96 protein-7 peptide complex. With 7 peptide, the use of Freund's adjuvant or chromalum resulted in a significant gain in immunological activity, and Freund's adjuvant was more effective than chromalum.

Therefore, subcutaneous injection was used in immunization. 7 peptide was dissolved in buffer solution (90% PBS, 10% DMSO/0.1% TFA), and gp96-7 peptide complex was dissolved in PBS. The solution was agitated vigorously for 1 minute before administration. For each mouse, the immunizing dose of the 7 peptide was 0.2 nmol, 2 nmol and 20 nmol. Emulsify the peptide with Freund's adjuvant before administering it to the mice. The immunizing dose of gp96-7 peptide complex was 0.01 nmol, 0.05 nmol and 0.50 nmol, and nape subcutaneous injection was used to immunize. This was followed up with a second injection one week after the first one. Cytotoxicity analysis was carried out 7 days later. For each injection, ten mice were used.

Example 11 Cytotoxicity (CTL) Analysis

Seven days after the immunization of the mice, about 3×10^7 spleen cells were collected from each mouse, and a suspension of such cells was made in a culture media containing 10mM HEPES buffer solution, 5×10^{-6} mercaptoethanol, antibiotics and 10% (V/V) FCS. The suspension was placed in complete culture medium for culture at 37°C. The same culture flask also contained radiated homologous LPS-simulated B lymphocytes (3:1) and 1 μg/ml of peptide. Six days later, spleen cells were collected for a four-hour standard ^{51}Cr release experiment (for details, see Kuhrober, A, et al. 1997. International Immunology, 9(8):1203-1212) to measure cytotoxic activity. In short, the target cells were sensitized with 10 μg/ml of peptide at 37°C for 30 minutes, then various quantities of effector cells were added. The reaction system was 100 μl of complete culture medium. After 4 hours of culture at 37°C, supernatant was collected to measure specific cleavage percentage.

The ^{51}Cr release experiment showed that both the 7 peptide and the gp96-7 peptide complex stimulated the mice to generate specific cytotoxic T lymphocyte, but the immunogenicity of the gp96-7 peptide complex was over 200 times greater than that of the 7 peptide. The use of 0.1 nmol (about 10 μg) of the gp96-7 peptide complex to immunize a mouse induced strong cellular immune response from the organism, and the measurement of cytotoxicity indicated more than 60% cleavage of the complex in target cells. The experiment showed that the gp96-7 peptide complex was a potential candidate for new drugs for treating HBV infection and HBV-infected liver cancer.

Example 12 Measurement of immunological activity of other gp96-polypeptide complexes

In addition to the 7 peptides described above, we combined in vitro six other HBV antigen polypeptides with gp96 and measured the immunological activity of complexes obtained. The six antigen polypeptides were: (1) core antigen polypeptide "YVNTNMGLK" (the 88-96 fragment of the core protein nucleic acid sequence); (2) core antigen polypeptide "STLPETTVVRR" (the 141-151 fragment of the core protein nucleic acid sequence); core antigen polypeptide "FLPSDFFPSV" (the 18-27 fragment of the core protein nucleic acid sequence); (4) surface antigen polypeptide "IPIPSSWAF" (the 313-321 fragment of the surface protein nucleic acid sequence); (5) surface antigen polypeptide "WLSLLVPFV" (the 355-343 fragment of the surface protein nucleic acid sequence); and (6) polymerase antigen polypeptide "FLLSLGIHL" (the 575-583 fragment of the polymerase protein nucleic acid sequence). These 6 polypeptides were artificially synthesized and each of them was combined in vitro with gp96, respectively, under the optimal reaction system in Example 8. All six resultant polypeptides showed a high affinity to gp96. For each of the

six polypeptides, the equilibrium constant K was measured, and results showed that all such Ks were greater than 5.

The said six HBV antigen polypeptide-gp96 complexes and the fusion proteins formed between gp96 and each of the said seven kinds of HBV antigen polypeptides were used to immunize mice in the method described in Example 10. The method described in Example 11 was used to perform CTL analysis on the said six complexes, respectively. The ^{51}Cr release experiment showed that all of the six HBV antigen polypeptide-gp96 complexes can stimulate the mice to generate specific cytotoxic T lymphocytes, and the immunogenicity of the six polypeptide-gp96 complexes was 150-300 times more than when a polypeptide was administered independently. The use of 0.1 nmol (about 10 μg) to immunize a mouse induced a strong cellular immune response from the organism, and the measurement of cytotoxicity of the six polypeptide-gp96 complexes indicated 60%-85% cleavage of the complex in target cells.

The results of the above experiment showed that the complexes formed by in vitro combination of the gp96 with the HBV antigen polypeptides were potential candidates for a new drug for the treatment of HBV infection and HBV-infected liver cancer. Due to limitation on the ability to carry out experiments, it was impossible for this invention patent to perform in vitro combination and measure the immunological activity of every HBV antigen polypeptide. Alternatively, we chose to focus our study on seven representative HBV core antigens, surface antigens and polymerases, combined them in vitro with gp96 and measured their immunological activity. Many experiments showed that all complexes formed between gp96 and these HBV antigen polypeptides stimulated strong immune response from mice, and gp96 may serve as an excellent new adjuvant for HBV antigen polypeptides, and was a potential candidate for a novel therapeutic vaccine. Therefore, the combination of any HBV antigen polypeptide other than the above said seven HBV antigen polypeptides with gp96 should be covered as novel vaccines under this invention.

Example 13 Gene cloning and expression of heat shock protein hsp78

The RNA isolation kit (Catrimox-14^{TM} RNA Isolation Kit Ver. 2.11) manufactured by TaKaRa Biotechnology (Dalian) Co., Ltd. was used to isolate the total RNA from 200 mg of human liver cancer tissue using the guanidinium isothiocyanate method. Instructions provided with the isolation kit were followed in the process.

Reverse transcriptase-polymerase chain reaction (RT-PCR) was then employed to clone hsp78, a member of the human hsp70 family. The RT-PCR Kit manufactured by TaKaRa Biotechnology (Dalian) Co., Ltd. was used and instructions provided with the isolation kit were followed in the process.

The following primers were used:
Primer 1: GG GGATCC ATG AAG TTC ACT GTG GTG GCG GCG
Primer 2: GG GTCGAC CTA CTA CTC ATC TTT TTC TGA TGT

PCR conditions: 4 minutes at 94°C; 50 seconds at 94°C, 50 seconds at 55°C, 2 minutes at 72°C, for 30 cycles; 5 minutes at 72°C.

The PCR product was a fragment that has a length of about 2.0 kb. Sequencing was performed on the fragment amplified by artificially introducing two restriction enzyme sites, for BamHI and SacII, to the 5' end and the 3' end. It was found that the hsp78 gene contained 1965 bp, and the base sequencing was consistent with reported results (Mech. Ageing Dev. 1998, 104 (2): 149-158).

The amplified fragment, after restriction using BamHI and SacIT enzymes, was linked to

the expression vector PET30a(+) and transformed to colibacillus BL21. At the same time, nucleic acid sequences associated with the following peptides were artificially synthesized: "YVNTNMG"; "YVNTNMGLK"; "STLPETTVVRR"; "FLPSDFFPSV"; "IPIPSSWAF"; "WLSLLVPFV"; "FLLSLGIHL". In addition, a BglII restrictionsite was introduced to the two ends of each of the said nucleic acid sequences, and the sequence was then inserted into the vector pET30a (+) containing gp78.

Expression occurs after inducing the vector with 1mM IPTG for 4 hours. The 10% SDS-PAGE electrophoresis method and the eCoomassie brilliant blue staining were used to measure the molecular weight of the expression product, and the result was about 80 kDa, which was basically consistent with the theoretical value.

Affinity chromatography was performed on the expression product with ADP-Agarose affinity chromatography (by Sigma) and the expression product was purified through MonoQ (by Pharmacia) anionic chromatography medium. The 10% SDS-PAGE electrophoresis method and silver staining (5 μg of sample in electrophoresis) were used to measure the purity. The purity of the protein was more than 95%. Western analysis was then performed on the expressed hsp78 and on its fusion protein, using hsp70 monoclonal antibody (by Sigma).

Example 14 Measurement of the immunogenicity of he hsp78-polypeptide complexes

The hsp78 was combined with each of the seven polypeptides in vitro into complexes under the same optimal reaction system as for the one gp96 (see Example 8), and these complexes and the fusion proteins formed by hsp78 and each of the seven polypeptides were used to immunize mice. The way of immunization, immunizing dose and immunization process were the same as those for gp96 (see Example 10). CTL analysis was the same as that for gp96 (See Example 11). The ^{51}Cr release experiment showed that the hsp78-7 peptide complexes stimulated the mice to generate specific cytotoxic T lymphocytes, and the immunogenicity of the hsp78-7 peptide complexes was over 150 times greater than when 7 peptide was administered independently. The use of 0.1 nmol (about 10 μg) to immunize a mouse induced a strong cellular immune response from the organism, and the measurement of cytotoxicity indicates 60%-85% cleavage of the complex in target cells. Experiments showed that the hsp78-7 peptide complexes may be further developed into a new drug for the treatment of HBV infection and HBV-infected liver cancer.

Claims

1. A complex formed between an HBV antigen and heat shock protein gp96 or hsp78.

2. The said complex in Claim 1, wherein the nucleic acid sequence of the said HBV antigen may be YVNTNMG, YVNVNMG, YVNTNMGLK, STLPETTVVRR, FLPSDFFPSV, IPIPSSWAF, WLSLLVPFV, FLLSLGIHL and/or one of their variants.

3. The said complex in Claim 1, wherein the heat shock protein was non-covalently bound to the HBV antigen, or the heat shock protein was covalently bound to the HBV antigen to form fusion protein.

4. A method for preparing the complexes formed by the HBV antigen and heat shock protein gp96 or hsp78. In the method, 0.1-0.15 μ mol/L of gp96 protein reacts with 2.5-3.5 μ mol/L of HBV antigen for 10-30 minutes at 30-39°C in a low salt buffer solution, whose concentration was not greater than 100 mmol/L and which contains 5-10% (v/v) glycerine.

5. The said method in Claim 4, wherein the reaction temperature was 37°C and the reaction time was 15 minutes.

6. The said method in Claim 4, wherein the concentration of the gp96 protein and the hsp78 was both 0.12 μ mol/L, and the concentration of HBV antigen was 3.0 μ mol/L.

7. The use of the said complexes formed between the HBV antigen and the heat shock protein gp96 or hsp78 in Claim 1 in preparing therapeutic vaccines for treating hepatitis B and the secondary liver cancer associated with hepatitis B.

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0.2 nmol peptide

2 nmol peptide

20 nmol peptide

0.1 nmol gp96-peptide complex

Effector Cells: Target Cells

Figure 1